Evaluation of an Anti-Inflammatory Factor Derived from Hyperimmunized Cows[1] (42832)

W. E. OWENS AND S. C. NICKERSON

Mastitis Research Laboratory, Hill Farm Research Station, Louisiana Agricultural Experiment Station, Louisiana State University Agricultural Center, Homer, Louisiana 71040

Abstract. **An anti-inflammatory factor isolated from milk of hyperimmunized cows was analyzed *in vitro* and *in vivo*. Macrophages collected from lacteal secretions of a unimmunized nonlactating cow showed increased ability to kill phagocytosed *Staphylococcus aureus* when incubated with the anti-inflammatory factor. Mice injected intraperitoneally with 10 mg/kg of anti-inflammatory factor demonstrated an increased LD_{50} to *S. aureus* when challenged intraperitoneally. Injected mice also demonstrated significantly ($P < 0.05$) less mammary inflammation and involution and increased clearance of *S. aureus* when challenged intramammarily. Quantitative histologic analysis of mammary tissues from mice injected with anti-inflammatory factor demonstrated significantly ($P < 0.05$) more lumen, less interalveolar connective tissue, and less leukocytic infiltration compared with control mice. Mammary glands of mice injected with anti-inflammatory factor and challenged with *S. aureus* also contained fewer colony-forming units than control mice. The product appeared to exert its effect on the nonspecific defense system via modulation of leukocyte function.**

[P.S.E.B.M. 1989, Vol 190]

Despite considerable strides in control of bovine mastitis during the past 20 years, this disease continues to inflict a significant economic loss on the United States dairy industry, exceeding 2 billion dollars annually (1). The economic importance of this disease continues to stimulate a variety of investigations for more efficient means of control. Although new methods and products for treatment are needed, common sense dictates that prevention of mastitis through sound management and enhancement of natural defenses is the preferred solution. Work is in progress on a variety of specific and nonspecific vaccines and immune modulators in attempts to modify the cow's natural ability to ward off intramammary infection.

Vaccine research has met with limited success. Evidence indicates that vaccination against *Staphylococcus aureus* can lessen the severity of mastitis and increase the spontaneous cure rate (2). Research has also been directed at nonspecific stimulation of the mammary gland leukocyte response using intramammary devices (3, 4). These devices have been shown to stimulate nonspecific leukocytosis into the gland and teat cisterns, increasing the number of phagocytes available to combat invading organisms. Additional research is being initiated on immunomodulators to enhance nonspecific resistance to invading organisms (5). A number of monosaccharides and polysaccharides have been shown to have immunomodulatory activity. Glucan from yeast and several plant glycans have been shown to enhance macrophage activity and increase resistance to bacterial challenge *in vivo* (6–8). Bacterial lipopolysaccharide, Bacille bilié de Calmette-Guerin, and *Corynebacterium parvum* are immunostimulators that appear to function by modulating macrophage activity (9, 10).

Recently, a novel anti-inflammatory factor (AIF) was isolated from dairy cows following hyperimmunization with a multivalent vaccine. The compound (Stolle Research and Development Corp., Lebanon, OH) is a low molecular weight (<10,000 daltons) substance consisting primarily of carbohydrates with traces of 18-carbon fatty acids. This research describes initial evaluation of the product for *in vitro* and *in vivo* en-

[1] Approved for publication by the Director of the Louisiana Agricultural Experiment Station as manuscript 88-80-2292.

Received May 18, 1988. [P.S.E.B.M. 1989, Vol 190]
Accepted September 16, 1988.

0037-9727/89/1901-0079$2.00/0

hancement of nonspecific resistance to infection with *S. aureus* Newbould 305.

Materials and Methods

Macrophage Assay. Mammary gland macrophages were collected from a nonlactating Jersey cow by intramammary infusion of 50 ml of pyrogen-free saline followed by gentle massage. Lacteal secretions containing macrophages were collected into 20 ml of ice-cold Hanks' balanced salt solution (HBSS) (Sigma Chemical Co., St. Louis, MO) to a total volume of 50 ml. Cells were centrifuged, washed twice in ice-cold HBSS, and resuspended in 10 ml of HBSS. Cell concentration and viability were determined by counting a trypan blue stained preparation in a hemacytometer. Cell types and percentage of macrophages were determined by microscopic evaluation of stained smears of the stock preparation. For the assay, cell concentration was adjusted to contain approximately 10^5 macrophages/ml. The initial collection and wash resulted in a cell preparation containing 40–60% macrophages, with the balance being primarily lymphocytes. This preparation was dispensed in 1-ml aliquots to 4-well tissue chamber slides (Lab Tek, Miles Scientific, Naperville, IL). Cells were allowed to adhere for 30 min at 37°C. After adherence, wells were washed three times with warm HBSS (37°C) to remove nonadherent cells, resulting in a preparation containing approximately 85% macrophages with the balance being primarily lymphocytes. After the final wash, HBSS was replaced with Medium 199 (Sigma) supplemented with 0.68 m*M* glutamine and 25 m*M* Hepes (Sigma); the AIF was added to wells in concentrations ranging from 0.1 to 100 μg/ml. Cells were incubated with AIF for 24 hr at 37°C with 5% CO_2. After incubation, Medium 199 was removed and cells were washed three times with HBSS. Opsonized *S. aureus* Newbould 305 (ATCC 29740) was added to each well at a concentration of 10^5 colony-forming units (CFU)/well. Bacteria were opsonized by prior incubation with 10% normal bovine serum for 30 min. After 2- or 4-hr incubation, cells were washed twice in HBSS and 5 units/ml of lysostaphin were added to lyse extracellular *S. aureus*. After 20-min incubation, cells were washed twice with HBSS. Wells for bacteriologic examination were treated with 0.05% saponin to lyse macrophages. The well contents were then aspirated and the number of viable *S. aureus* was determined by the standard plate count method. Wells used for microscopic evaluation were washed in HBSS and then stained with Wright stain. Stained slide wells were examined microscopically and the percentage of macrophages ingesting *S. aureus* was determined by direct microscopic count. One hundred macrophages per slide were examined.

Determination of LD_{50}. The effect of AIF on the LD_{50} of *S. aureus* Newbould 305 for mice was determined using the method of Reed and Muench (11). Mice were injected with 10 or 100 mg/kg of AIF for 7 days prior to challenge. Three groups of six mice were challenged with 1.2×10^{10}, 1.2×10^9, or 1.2×10^8 CFU of the organism. Survival was determined at 24 hr and the LD_{50} calculated.

Mouse Mastitis Model. The mouse mastitis model of Anderson (12) was used to determine the effect of AIF on experimentally infected mammary glands of lactating mice. Two groups of three lactating mice were injected intraperitoneally with 10 mg/kg AIF suspended in 0.1 ml of HBSS; similar control groups received HBSS only. Mice were injected daily for 3 to 4 days prior to parturition and for 3 to 4 days thereafter for a total of 7 days. On Day 7, a control and test group received intramammary injection of 3×10^3 CFU of *S. aureus* Newbould 305 per gland in glands R4 and L4. An additional control and test group received 2.5×10^2 CFU/gland. After 24 hr, mice were sacrificed by cervical dislocation and R4 and L4 glands removed aseptically. Glands were bisected and one half was processed for histology and the other half were homogenized in 9 ml of sterile saline. Tissues used for histologic assessment were processed for light microscopic examination as described in (8). Briefly, tissue samples were fixed for 24 hr in Bouin's fluid. Specimens (0.5–1.0 cm^3) were prepared for sectioning by infiltration and embedding in Paraplast (American Scientific Products, McGraw Park, IL). Tissue sections (5 μm) were stained with hematoxylin and eosin and observed using a Zeiss Standard 18 research microscope. The saline homogenate was plated to bovine blood agar to determine CFU of *S. aureus* per gland.

Morphometric Analysis. Quantitative morphometric analysis was used to determine percentage of mammary tissue area composed of epithelium, alveolar lumen, and interalveolar connective tissue. For each tissue sample, 100 contact points were counted per slide at a magnification of 640×. A reference grid in the microscope ocular provided fixed points used in the counting process. Tissue specimens of mammary parenchyma were also examined for the presence of leukocyte infiltration. Prevalence of these cells was quantified at 640× in 10 randomly selected microscope fields per slide and assigned a score where 1 = few or no leukocytes, 2 = moderate leukocyte infiltration, and 3 = intense leukocyte infiltration. Data were analyzed by analysis of variance using the general linear models procedure. Duncan's multiple range test was used to detect differences among means. Effects of treatment and dose were determined on the occurrence of the histologic components measured.

Results

Macrophage Assay. No difference was observed in the number of macrophages containing phagocytosed bacteria from control wells and wells receiving AIF. Approximately 25% of macrophages contained

bacteria in control and test wells at 2 hr and 40–60% at 4 hr. There was, however, a difference in the number of viable internalized bacteria between control and test wells. Table I shows that control wells contained an average of 12×10^3 CFU of *S. aureus* after 2 hr and 6.5×10^3 after 4-hr incubation. Test wells containing 10 and 100 μg of AIF contained 9.8×10^3 and 6.5×10^3 CFU, respectively, at 2 hr. Wells containing 100 μg of AIF yielded 1.4×10^3 CFU after 4-hr incubation. Wells receiving 0.1 and 1 μg were similar to controls.

Table I. Viable *S. aureus* (CFU/ml × 10^3) Present within Macrophages after Incubation with Various Concentrations of AIF

Incubation time (hr)	Concentration of AIF (μg/ml)				
	0	0.1	1	10	100
2	12.0	11.0	12.0	9.8	6.5
4	6.5	—[a]	7.6	—[a]	1.4

[a] Assay not performed.

Table II. Viable *S. aureus* (CFU/ml × 10^3) Isolated from Mammary Gland Homogenates of Mice Injected with 10 mg/kg of AIF for 7 days and challenged intramammarily with 2.5×10^2 CFU of *S. aureus*

Control[a]			AIF treated		
Mouse	Right gland	Left gland	Mouse	Right gland	Left gland
A	4.0	—	D	1.2	.9
B	6.5	3.0	E	2.1	1.4
C	3.4	4.1	F	0.8	2.0
Mean CFU/ gland	4.2			1.4	

[a] Control mice were injected ip with 0.1 ml of HBSS.

LD_{50} Assay. Mice receiving 10 mg/kg of AIF demonstrated an apparent increased tolerance to *S. aureus* challenge compared with control mice. The LD_{50} was increased from $1.2 \times 10^{9.5}$ to $>1.2 \times 10^{10}$ CFU. Mice receiving 100 mg/kg did not demonstrate increased tolerance to bacterial challenge.

Mouse Mastitis Challenge. Table II shows mouse challenge data and demonstrates the increase in tolerance to *S. aureus* mastitis induced by 10 mg/kg of AIF. Attempts to quantify bacterial numbers from mice challenged with 3×10^3 CFU revealed no detectable differences between control and test mice due to the large numbers of organisms present. A repeat of the experiment with reduced numbers of challenge organisms did reveal a difference in bacterial numbers between control and test mice. However, the differences were not statistically significant. Control glands contained an average of 4.2×10^3 CFU while AIF-treated mice contained 1.4×10^3 CFU/gland (Table II).

Lactating mice receiving daily injections of 10 mg/kg of AIF demonstrated a markedly different response to intramammary challenge than did control animals. Histologic examination of mammary gland tissue obtained 24-hr after inoculation from control mice showed marked inflammation and involution of the secretory parenchyma in response to bacterial challenge. Tissues exhibited a limited alveolar luminal area, most of which was filled with leukocytes, and a large interalveolar stromal area composed primarily of adipocytes and infiltrating leukocytes (Fig. 1). The alveolar epithelium was highly vacuolated but showed no signs of necrosis (Fig. 2). In contrast, mammary tissues from AIF-treated mice exhibited far less inflammation and

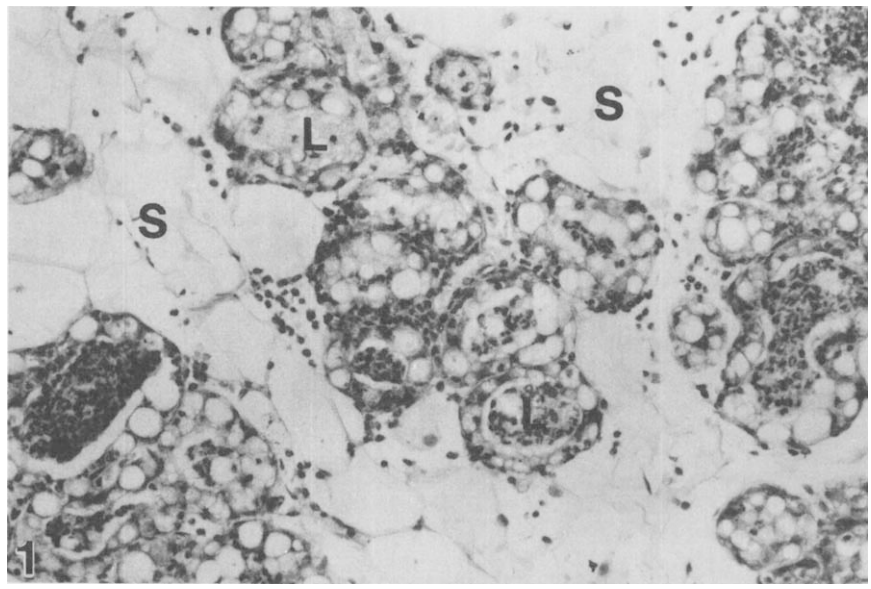

Figure 1. Mammary tissue from a control mouse 24 hr after challenge with 3×10^3 CFU of *S. aureus* illustrating limited alveolar luminal area (L) and large interalveolar stroma (S) (original magnification ×210).

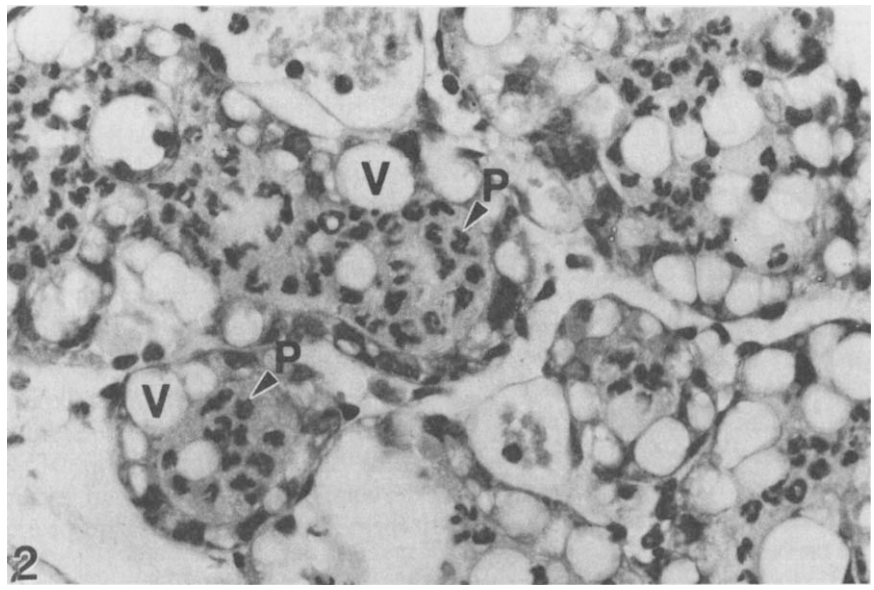

Figure 2. Higher magnification of tissue area similar to that in Figure 1 illustrating numerous PMN (P) in alveolar lumina and large vacuoles (V) in epithelium (original magnification ×500).

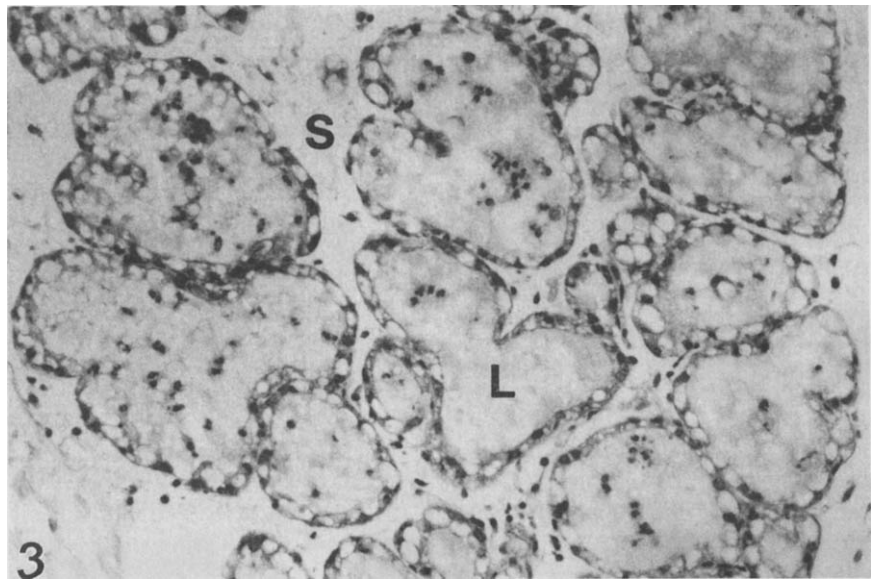

Figure 3. Mammary tissue from an AIF-treated mouse, 24 hr after challenge with 3×10^3 CFU of *S. aureus* illustrating large alveolar luminal area (L) and limited stroma (S) (original magnification ×210).

involution. Examination of parenchymal tissues revealed larger luminal areas and smaller stromal areas compared with control tissues. Moderate leukocyte infiltration was observed in both areas (Fig. 3). The alveolar epithelium was less vacuolated in comparison with control tissues (Fig. 4).

Morphometric comparison of AIF-treated and control glands revealed marked differences in parenchymal components and degree of leukocyte infiltration (Table III). AIF-treated mice exhibited significantly more ($P < 0.05$) alveolar luminal area (49.2% vs 25.6%), significantly less ($P < 0.05$) interalveolar stromal area (20% vs 42%), and significantly less ($P < 0.05$) leukocyte infiltration (1.6% vs 2.9%) compared with controls. Percentages of alveolar epithelial cells were not different between treatment groups.

Mammary tissue from AIF-treated mice receiving the lower dose exhibited a larger luminal area and more flattened epithelium (Fig. 5; compare with Fig. 3). Mammary glands of control mice receiving the lower dose exhibited more alveoli, larger luminal area, and fewer leukocytes (Fig. 6; compare with Fig. 1). Mice receiving the larger intramammary challenge dose (3×10^3 CFU) had more pronounced differences among AIF-treated and control groups than mice receiving 2.5×10^2 CFU (Figs. 7, 8 and Table IV). Although the

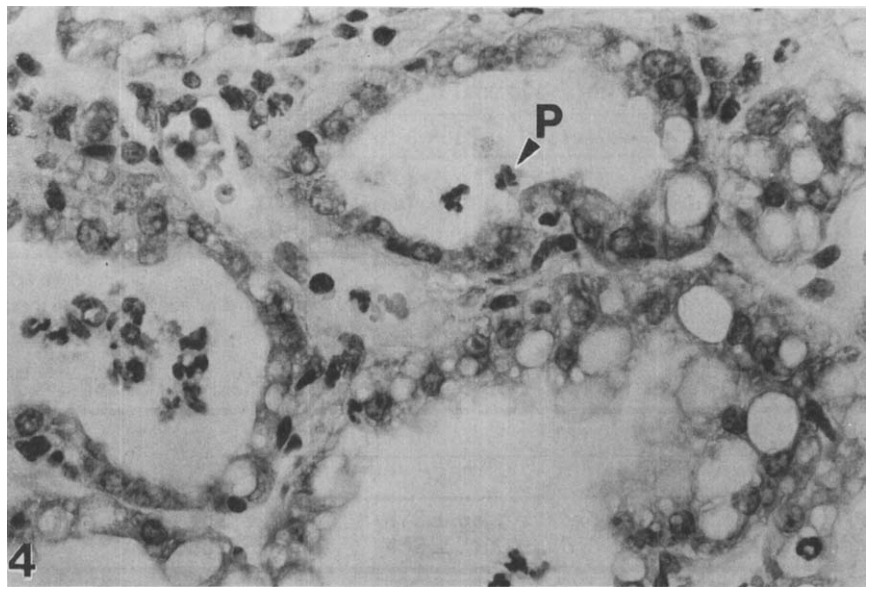

Figure 4. Higher magnification of tissue area similar to that in Figure 3 illustrating moderate PMN (P) infiltration into luminal and stromal areas. Alveolar epithelium is less vacuolated than that observed in Figure 2 (original magnification ×500).

Table III. Effect of 10 mg/kg of AIF Treatment on the Mammary Histologic Response[a] to *S. aureus* Intramammary Challenge

Histologic parameter	Treatment[b]	
	AIF	Control
Epithelium	30.8[c]	32.4[c]
Lumen	49.2[d]	25.6[c]
Stroma	20.0[c]	42.0[d]
Leukocyte infiltration	1.6[c]	2.9[d]

[a] Data on epithelium, lumen, and stroma are expressed as percentage of tissue area. Leukocyte infiltration was assigned a score where 1 = few or no leukocytes, 2 = moderate leukocyte infiltration, and 3 = intense leukocyte infiltration.
[b] Data for Doses 1 (3×10^3 CFU) and 2 (2.5×10^2 CFU) are combined.
[c,d] Values in a row with different superscripts differ $P < 0.05$.

epithelial area did not change between treated and control groups, there was significantly more ($P < 0.05$) area assigned to epithelium in mice receiving the 3×10^3 CFU dose than the 2.5×10^2 CFU dose. Luminal area was significantly lower ($P < 0.05$) in both AIF-treated and control groups for the 3×10^3 CFU dose compared with the 2.5×10^2 CFU dose. Stromal area was significantly higher ($P < 0.05$) in the control vs treated group, but there were no significant effects of dose on either group. Although dose had no significant effect on leukocyte infiltration in AIF-treated mice, infiltration was significantly higher ($P < 0.05$) in the 3×10^3 vs 2.5×10^2 CFU dose of control mice. Parenchymal areas from mice challenged intramammarily with the 3×10^3 CFU dose contained large ducts occluded with leukocytes (Fig. 7). Cocci were observed free within alveolar lumina, but most were found within polymorphonuclear leukocytes (PMN) (Fig. 8).

Discussion

There are many substances that have the ability to modulate nonspecific immunity including prostaglandins, transfer factor, interleukin 2, lymphokines, monokines, lipopolysaccharide, and glycans. The carbohydrate nature of the AIF suggests a possible similarity to other carbohydrates shown to have immunostimulatory properties. Selgelid *et al.* (7) demonstrated stimulation of mouse macrophages with a number of glycans of plant and yeast origin. Glucan, a β-1,3-polyglucose from yeast has been shown to have significant effects on both humoral and cellular immunity particularly via macrophage modulation (6–8). A recent study by Buddle *et al.* (6) demonstrated a protective effect by glucan in ewes with experimentally induced *S. aureus* mastitis. Glucan was shown to stimulate ovine macrophages *in vitro* and increase resistance of ewes to *S. aureus* challenge *in vivo*. Preliminary data indicate that the AIF used in the present study may act in a similar manner.

The AIF derived from hyperimmunized cows succeeded in reducing inflammation and the number of viable organisms present in mammary glands of mice challenged with *S. aureus*. Morphologic changes observed in mammary glands of control mice 24 hr after challenge with *S. aureus* were similar to those observed by others using the same bacterial species (14, 15). The alveolar luminal area was reduced and the stromal area and leukocyte infiltration were increased, suggesting a reduction in secretory activity and involution of parenchymal tissues. In contrast, inflammation and involu-

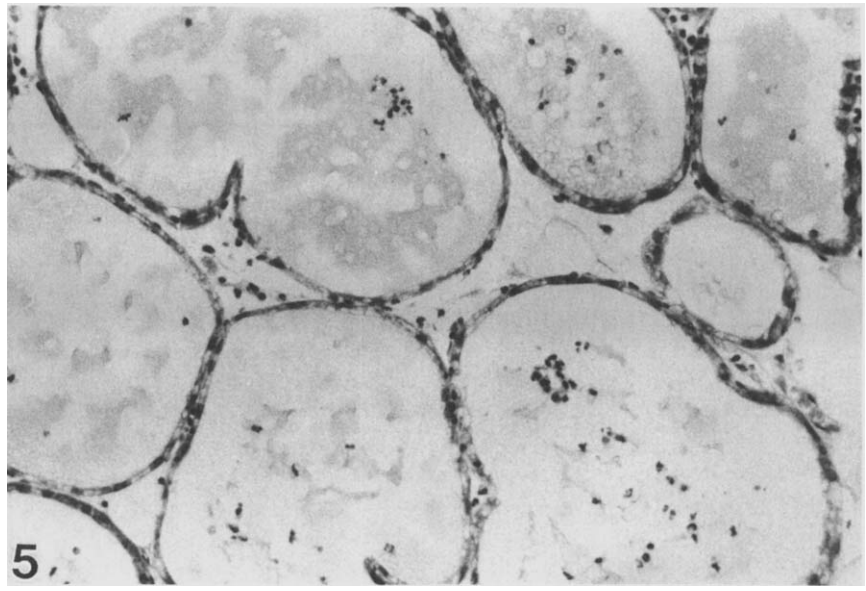

Figure 5. Mammary tissue from AIF-treated mouse challenged with 2.5×10^2 CFU of *S. aureus* illustrating distended alveolar lumina and flattened epithelium (original magnification ×500).

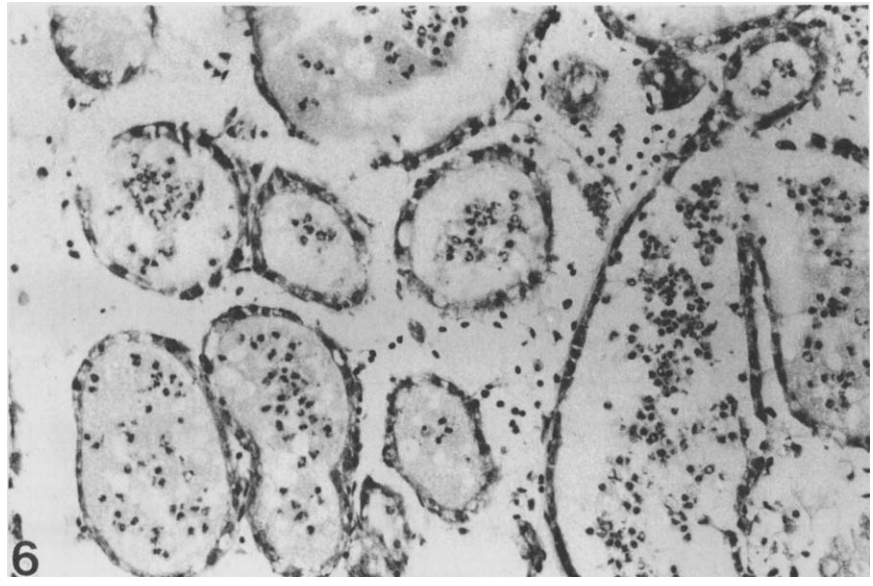

Figure 6. Mammary tissue from control mouse challenged with 2.5×10^2 CFU of *S. aureus* illustrating less luminal area, more stroma, and greater leukocyte infiltration compared with Figure 5 (original magnification ×500).

tion as well as bacterial numbers were reduced in AIF-treated mice, indicating that these mice were better able to clear the *S. aureus* challenge and maintain normal secretory activity.

In the present study, macrophage cultures incubated with AIF were better able to kill phagocytosed bacteria than controls. Additional research in a smoking rat lung model suggests that alveolar macrophages of rats fed AIF were better able to phagocytose zymosan particles than those of control animals (W. Wilborn, personal communication). Macrophages are the predominant leukocyte type in milk from normal, uninfected mammary glands, and the first bacteria-cell interaction may involve these cells (16). It has been suggested that macrophages in normal milk function as monitors of bacterial infection and phagocytic activity, leading to the rapid recruitment of PMN (17). The latter cell type constitutes the first line of defense once bacteria have entered the mammary gland (18).

Work with intramammary devices (3, 4, 19) demonstrated that sustained contact with cisternal tissues resulted in an increased influx of PMN into these areas, thereby increasing the resistance of the gland to infection. Other workers have suggested that recruitment of

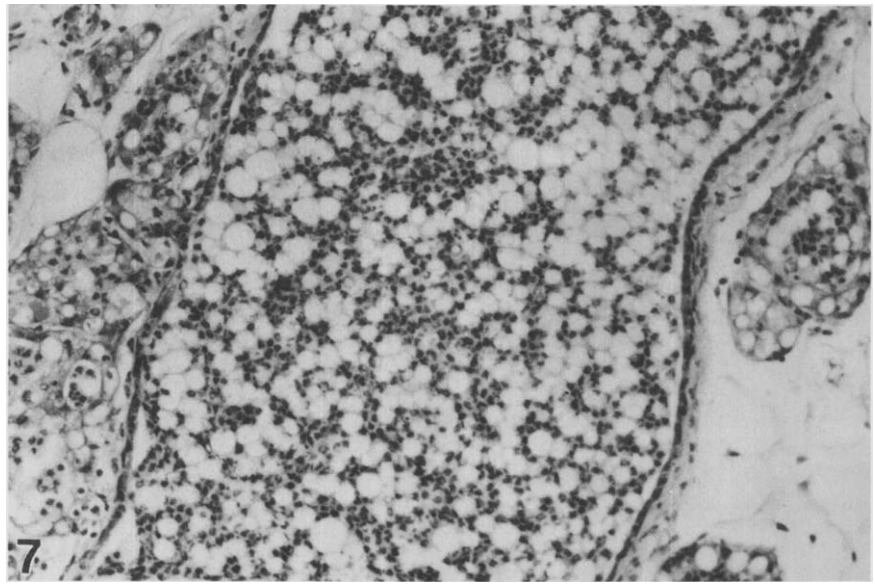

Figure 7. Mammary tissue from control mouse 24 hr after challenge with 3×10^3 CFU of *S. aureus* illustrating large duct occluded with PMN (original magnification ×500).

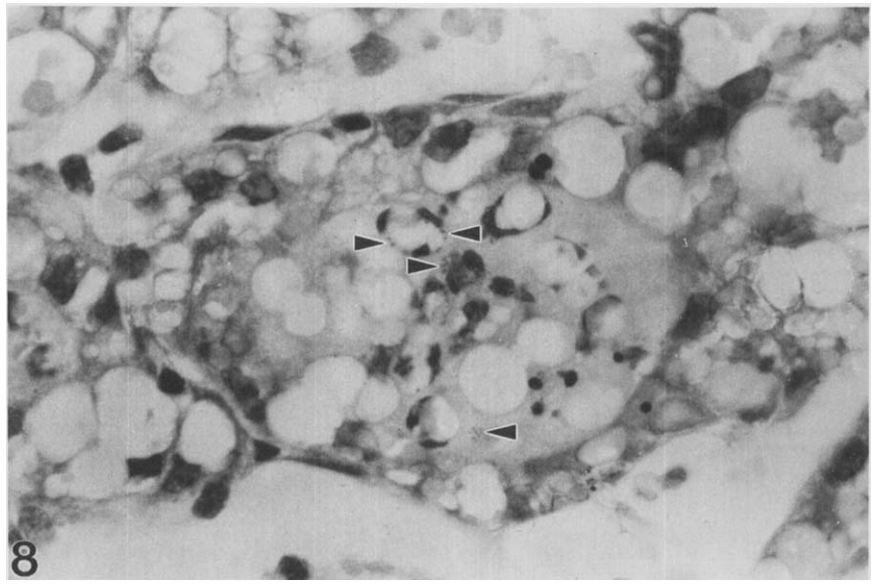

Figure 8. Higher magnification of alveolus in tissue area similar to that in Figure 7 illustrating cocci (arrowheads) free in lumen and within PMN (original magnification ×825).

Table IV. Effect of 10 mg/kg of AIF Treatment on the Mammary Histologic Response[a] to *S. aureus* Challenge with 3×10^3 and 2.5×10^2 CFU/Gland

Histologic parameter	Treatment			
	AIF		Control	
	3×10^3	2.5×10^2	3×10^3	2.5×10^2
Epithelium	35.4[b]	23.8[c]	38.1[b]	22.2[c]
Lumen	44.3[b]	56.7[c]	13.8[d]	47.0[c]
Stroma	20.3[b]	19.5[b]	48.1[c]	31.0[c]
Leukocyte infiltration	1.7[b]	1.5[b]	3.0[c]	2.6[d]

[a] Data for epithelium, lumen, and stroma was expressed as percentage of tissue area. Leukocyte infiltration was assigned a score where 1 = few or no leukocytes, 2 = moderate leukocyte infiltration, and 3 = intense leukocyte infiltration

[b,c,d] Values in a row with different superscripts differ $P < 0.05$.

fresh PMN into the gland effectively increases the overall level of leukocyte activity (4). This fresh influx of new PMN replaces older less active cells, so that while individual cells may not be stimulated, the overall effect is to increase the level of PMN activity in the gland. The substance also had substantial anti-inflammatory activity as indicated by the significant reduction in inflammation present in mammary glands of AIF-treated mice. Research by others (18, 20) on a variety of anti-inflammatory compounds and their effect on PMN function indicated that some of these compounds, notably methylprednisolone and ibuprofen, were beneficial to bovine PMN function and may have limited milk losses during mammary inflammation. Although the AIF evaluated here is unrelated chemi-

cally to these anti-inflammatory agents, it may have similar potential for use in treatment of mastitis.

Additional research is needed to determine whether the substance can offer substantial protection against infection and to clarify the mechanisms involved in its action.

1. Eberhart RJ, Harmon RJ, Jasper DE, Natzke RP, Nickerson SC, Reneau JK, Row EH, Smith KL, Spencer SB. Current Concepts of Bovine Mastitis. The National Mastitis Council, pp6–8, 1987.
2. Pankey JW, Boddie NT, Watts JL, Nickerson SC. Evaluation of protein A and a commercial bacterin as vaccines against *Staphylococcus aureus* mastitis by experimental challenge. J Dairy Sci **68**:726–731, 1985.
3. Nickerson SC, Thompson WJ, Kortum WM, Boddie NT. Histological response of bovine mammary to an intracisternal device. J Dairy Sci **70**:687–695, 1987.
4. Paape MJ, Schultz WD, Guidry AJ, Kortum WM, Weinland BT. Effect of an intramammary polyethylene device on the concentration of leukocytes and immunoglobulins in milk and on the leukocyte response to *Escherichia coli* endotoxin and challenge exposure with *Staphylococcus aureus*. Am J Vet Res **42**:774–783, 1981.
5. Nickerson SC. Enhancing local udder immunity against *Staphylococcus aureus* with interleukin-2 (IL-2). Page 103 *in* Proceedings of the 27th Annual Meeting of the National Mastitis Council, Inc. Reno, NV, 1988.
6. Buddle BM, Pulford HD, Ralston M. Protective effect of glucan against experimentally induced staphylococcal mastitis in ewes. Vet Micro **16**:67–76, 1988.
7. Selgelid R, Bogwald J, Lundwall A. Glycan stimulation of macrophages in vitro. Exp Cell Res **131**: 121–129, 1981.
8. Wooles WR, DiLuzio NR. Reticuloendothelial function and the immune response. Science **142**:1078–1080, 1963.
9. Adlam C, Broughton ES, Scott MT. Enhanced resistance of mice to infection with bacteria following pretreatment with *Corynebacterim parvum*. Nature **235**:219–230, 1972.
10. Poplack DG, Sher NA, Chaparas SD, Blaese RM. The effect of *mycobacterium bovis* (Bacillus Calmette-Guerin) on macrophage random migration, chemotoxis and pinocytosis. Cancer Res **36**:1233–1239, 1976.
11. Reed LJ, Muench H. A simple method of estimating fifty percent end points. Am J Hyg **27**:493–497, 1938.
12. Anderson JC. The contribution of the mouse mastitis model to our understanding of staphylococcal infection. In Jeljaszewicz J, Ed. Staphylococci and Staphylococcal Diseases. Stuttgart, Gustav Fischer Verlag, pp783–790, 1976.
13. Sordillo LM, Nickerson SC. Growth patterns and histochemical characterization of bovine mammary corpora amylacea. J Histochem Cytochem **34**:593–597, 1986.
14. Chandler RL. Experimental bacterial mastitis in the mouse. J Med Microbiol **3**:273–282, 1970.
15. Anderson JC, Chandler RL. Experimental staphylococcal mastitis in the mouse. Histological, ultrastructural and bacteriological changes caused by a virulent strain of *Staphylococcus aureus*. J. Comp Pathol **85**:499–510, 1975.
16. Jensen DL, Eberhart RF. Macrophages in bovine milk. Am J Vet Res **36**:619–624, 1975.
17. Craven N. Generation of neutrophil chemoattractants by phagocytosing bovine mammary macrophages. Res Vet Sci **35**:310–317, 1983.
18. Nickerson SC. Resistance mechanisms of the bovine udder: New implications for mastitis control at the teat end. J Am Vet Med Assoc **191**:1484–1488, 1987.
19. Paape MJ, Ziv G, Miller RH, Schultze WD. Update on the use of intramammary devices in the control of mastitis. Proceedings of the 25th Annual Meeting of the National Mastitis Council, Inc, Columbus, OH, p87, 1986.
20. Nickerson SC, Paape MJ, Harmon RJ, Ziv G. Mammary leukocyte response to drug therapy. J Dairy Sci **69**:1733–1742, 1986.